Exhibit B

Protein microarrays: A powerful tool to study cancer

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Protein microarrays offer a robust way of examining the cellular proteome. The use of protein arrays to study cancer will enable better understanding of the molecular events associated with tumor development and progression. The use of antigen and antibody arrays could revolutionize the area of biomarker discovery, deciphering biochemical and signaling pathways and pharmaceutical research. This review will focus on current developments in the area of protein microarrays and their application in the study of cancer.

Keywords Antibody array, antigen array, cancer, protein microarray

Introduction

Cancer is one of the leading causes of death in the modern world. Since the use of the term as early as 1600 BC by the Egyptians, who described the use of surgical, pharmacological, mechanical and magical treatments, this deadly disease has become the topic of intense scientific investigation. Approximately one in three individuals in the western world are affected by this disease [1]. Although cancer is often referred to as a single condition, it actually consists of more than one hundred different diseases, characterized by uncontrolled growth and spread of abnormal cells. Cancer can arise in many sites and behave differently depending on its site of origin. Recent advances in the study of cancer include the use of DNA microarrays to obtain gene profiles, which are used to diagnose the disease, predict its progression and design treatment regimens.

The human genome encodes as many as 30,000 to 50,000 proteins. The number could be considered much higher as protein expression is regulated at many stages by transcription, alternative splicing, translation, post-translation (phosphorylation, glycosylation and protein splicing), compartmentalization [2], and interaction with other proteins. Hence it becomes difficult to make linear comparisons between expression of transcript and protein function [3]. Although a wealth of gene expression data has been generated over the past decade encompassing a wide range of cancers [4-8], the area of oncoproteomics using high-throughput protein microarrays is still in its infancy. Current methods for studying protein-protein interactions rely on techniques such as the yeast two-hybrid system, co-immunoprecipitation and phage display [2], while protein quantitation in various biological samples is achieved using techniques such as one/ two-dimensional gel electrophoresis, immunoblotting, enzyme-linked immunosorbent assay (ELISA) and radioimmune assay. These methods either lack the dynamic range for detecting minor alterations in levels of proteins, or do not allow the study of global changes in protein expression [2]. Therefore, it is necessary to develop high-throughput technologies, similar to DNA microarrays, to study global changes in protein expression and function. The development of such high-throughput proteomic platforms to study cancer would effectively complement the existing genomic data and help the understanding of the pathophysiology, biochemical pathways and signaling networks that regulate the development, maintenance and progression of this deadly disease. Moreover, such understanding would enable the design of strategies for early diagnosis of, and therapies for cancer.

The development of high-throughput techniques to assay protein expression has been difficult. Unlike nucleic acids that interact with each other by complementary base pairing, protein-protein interaction depends on a number of factors that include three-dimensional conformation of the protein, pH, ionic strength, temperature and presence of inhibitors. Therefore, maintaining the protein in a functional state has been a major challenge. Proteins are also prone to non-specific interactions. Despite these issues, recent advances in high-throughput screening has led to the development of numerous chip-based assay systems for monitoring protein expression and activities in cells. Most of these rely on spotting either the antigen or antibody as baits on derivatized glass surfaces using high precision robotic arrayers. These chips are then probed with fluorescent-labeled probes containing purified proteins, antibodies, cell lysates or sera. The detection involves the use of a single fluorochrome or two fluorochromes with different emission spectra. In the former case, various internal standards are used to normalize the data across hybridizations. The latter method resembles spotted DNA arrays wherein the control sample and the treated samples are labeled with different fluorochromes [9] and the relative level of proteins in each of the samples is obtained. Such biochips enable high-throughput analysis of very low amounts of biological samples at relatively low cost and less time. This review describes the various advances made in the area of antigen/ antibody arrays and highlights their potential application in the study of cancer.

Antigen microarray

This involves spotting purified proteins on modified glass slides as bait to capture interacting partners from complex biological samples. Many groups have tested the sensitivity and specificity of antigen chips using labeled antibodies spiked in the milieu of large excess of extraneous proteins such as bovine serum albumin (BSA) and fetal calf serum (FCS) [10••,11••]. The presence of such an excess of non-specific protein mimics a biological sample in which the antigens of interest could be present in very low amounts. The antigen chip developed by our group was tested for its limit of detection by spotting different concentrations of rabbit IgG ranging from 1.6 to 50 pg [10••]. A linear range of detection of the spotted antigen was obtained using Cy5-labeled anti-

rabbit IgG, with sensitivity close to 6.25 pg of spotted IgG. Also, detection of the spotted protein on the antigen chip was highly specific when probed with a labeled mixture of cognate antibodies in large excess of non-specific proteins (1% BSA). Such a high degree of sensitivity and specificity enables the use of antigen arrays to study changes in protein levels in complex biological samples. In a parallel study, Haab *et al* tested the sensitivity and specificity of their antigen chips using 115 antigen-antibody pairs [11••]. The validation used six different mixtures of antibody, each labeled with Cy5 dye against a constant concentration of Cy3-labeled reference mix. The color of the antigen spots on the array varied concordantly with change in the concentration of their binding partners in the probe mixture. The sensitivity of the antigen chip was as low as 100 pg/ ml, even in the presence of 10- to 100-fold excess of FCS. Similarly, in an elegant set of experiments addressing the specificity of an antigen chip, MacBeath *et al* demonstrated that the binding of labeled FK-506-binding protein (FKBP)-12 to the rapamycin binding domain of spotted FKBP-rapamycin associated protein (FRAP) occurred only in the presence of the cofactor rapamycin [12]. Such specificity of interaction of spotted proteins on an antigen chip has an immense potential for studying protein-protein interactions, enzyme-substrate interactions, etc, which play an important role in cancer.

One of the major concerns during fabrication of antigen arrays is the nature of the ideal substrate for spotting proteins. An ideal substrate should bind the capture molecules with high affinity, allow retention of their functional activity and give low autofluorescence [13]. There are many substrates available, many of which are based on various modifications of glass. These include poly-L-lysine-, aldehyde-, hydrogel-, polyacrylamide- and nitrocellulose-coated slides. Among these, the first four are thought to give high signal-to-noise ratio with low inter-field variations and attomole detection limit [13]. Similar results have been reported using reflective surfaces and non-gel coated plastic slides such as maxisorb [13]. However, nitrocellulose-coated surfaces seem to provide improved sensitivity and low signal-to-noise ratio [10••,14••,15••,16••]. One of the major drawbacks of using nitrocellulose-based support is the inability to direct the orientation of spotted proteins [14••]. This could be addressed using site-specific immobilization of proteins to substrates using generic ligands such as protein A/ G, biotin-avidin system [17] or derivatized metals like Ni-NTA [18]. These approaches also increase the sensitivity of detection by maintaining the functional activity of a large percentage of spotted proteins. Thus, an antigen chip enables high-throughput analysis of biological samples and could potentially replace conventional techniques such as the yeast two-hybrid, ELISA and immunoblot assays.

Applications

Among the many potential applications of the antigen chip, the important ones in the study of cancer proteomics include monitoring protein-protein interactions in cell lysates, detection of circulating antibodies in patient sera, identification of biomarkers in various biological samples and screening of large expression libraries (bacterial/ phage display) for novel interacting partners. In addition to the conventional antigen arrays mentioned above, innovations based on the same underlying principle have resulted in the development of a number of novel applications.

One such interesting application with potential importance in drug discovery involves the development of arrays to study ligands for G protein-coupled receptors (GPCRs). Fang *et al* developed membrane-based GPCR arrays by spotting membrane-associated suspensions on modified glass slides [19]. These membrane arrays were probed with fluorescently labeled ligands to determine binding constants for ligand-receptor interactions. Importantly, the binding constants for various ligand-receptor pairs obtained from the GPCR arrays were comparable to those reported using other methods. Also, using these receptor arrays, it is possible to screen for specific ligands to a group of receptors [19]. Such arrays have immense potential in screening various defects in the GPCR-mediated mitogenic signaling cascade that could be one of the causes of tumor formation. Moreover, GPCR arrays could also be used to identify potential ligands for the various orphan GPCRs, many of which could be targets for drug design.

Another relevant application of antigen arrays in the area of oncoproteomics is to monitor protein-protein interactions. These interactions regulate various vital processes like replication, transcription and translation. Cancer could result from a perturbation in any of these processes. Current methods used to study such interactions involve the use of a yeast two-hybrid system that is both labor-intensive and time consuming. However, antigen arrays offer the ability to study such interactions using high-throughput technology on a cost-effective scale. Thus, a biochip containing various interacting motifs could be used to screen for domains that are required for interaction between proteins. Since many of these protein-protein interactions could be deleterious, leading to tumorogenesis in many cases, knowledge of the interacting motifs could aid in designing inhibitors that may affect such interactions.

Arrays to monitor protein interaction domains in signaling molecules were developed by Espejo *et al*, consisting of glutathione-S-transferase-tagged recombinant proteins having stretches of various interacting domains like WW, H3, SH2, 14.3.3, FHA, PDZ, PH and FF [20•]. Using these 'motif chips', the potential interacting regions in the signaling molecule sam68 and core snRNP protein SmB were identified [20•].

The antigen chips mentioned in the above studies consist of purified recombinant proteins that function as bait for the capture of molecules from complex biological samples. However, such a system does not allow for population-based screening of samples that might be important in the area of biomarker discovery and monitoring effects of treatment regimens. These require comparison of multiple data points across many patient samples under similar experimental conditions. Unfortunately, none of the classical techniques provide such robustness. However, an antigen chip containing the entire repertoire of cellular proteins from a large number of patients could allow for such comparisons. Such an array, also termed reverse-phase protein microarray, could be used for screening large sample populations under identical experimental conditions. Moreover, since the amount of analyte required for such chip-based antigen arrays is lower than for other conventional techniques, it allows for screening of pure tumor cell populations obtained by laser capture

microdissection. Reverse-phase protein arrays allow screening of multiple proteins (biomarkers) among large number of samples using minute amounts of starting material, under identical experimental conditions. This was demonstrated by Pawaletz *et al* who used laser captured cells from different grades of prostate cancer to study the changes in expression of various molecular regulators governing the prosurvival pathway during the transition of prostate cells from a normal to an oncogenic state [15••]. Importantly, this method not only allowed for the detection of changes in the levels of protein *per se* but also enabled the monitoring of changes in their phosphorylation status. Results showed a strong positive correlation between phosphorylation of Akt and cancer progression, which is augmented by activation of the prosurvival pathway with a decrease in levels of phosphorylated ERK [15••].

In addition to its use in high-throughput screening of tumor markers in patient samples, reverse-phase protein arrays could also be used to detect autoantibodies against various cancer proteins (also termed humoral response). This is based on the assumption that the immune system could generate autoantibodies against various tumor-specific proteins that are considered 'foreign'. As many of these immunodominant proteins in the cancer cell might be present in very low abundance, one could consider generating a biochip consisting of fractionated cellular proteins. Such a chip containing the cancer proteome could be probed with sera from different patient populations to obtain a cancer-specific humoral response signature that might prove to be a sensitive marker for early sero-diagnosis of cancer. In this regard, spotting the fractionated proteins in a biochip format not only offers the advantage of screening multiple sera, but also the ability to detect humoral response using minute amounts of proteins resulting from fractionation. In one such approach, Madoz-Gurpide *et al* spotted fractionated proteins from lung adenocarcinoma cells and probed with antibodies directed against specific antigens [14••]. One of the advantages of analyzing the fractionated proteins in the biochip format as against the conventional 2D-electrophoresis is its ability to detect low molecular weight proteins and highly hydrophobic proteins [14••].

Parallel studies using a similar strategy were used to identify specific antibody repertoires generated as a result of humoral response during various diseased states [21•, 22••]. The antigen chips containing spotted microbial antigens, recombinant proteins, or immunodominant peptides were used to study humoral response against various microbial antigens [21•] or in sera of patients with autoimmune disorders [22••].

Reverse-phase technology adds a new dimension to the existing high-throughput formats. The power behind this system is its ability to analyze large numbers of clinical samples in minute quantities for perturbation in specific protein levels. In addition to its current utility, it could also be used to look for antigen biomarkers in patient sera for various disease states, antibody response in individuals undergoing diverse therapeutic regimens, screening expression libraries for protein-protein interactions, and screening hybridomas.

Antibody arrays

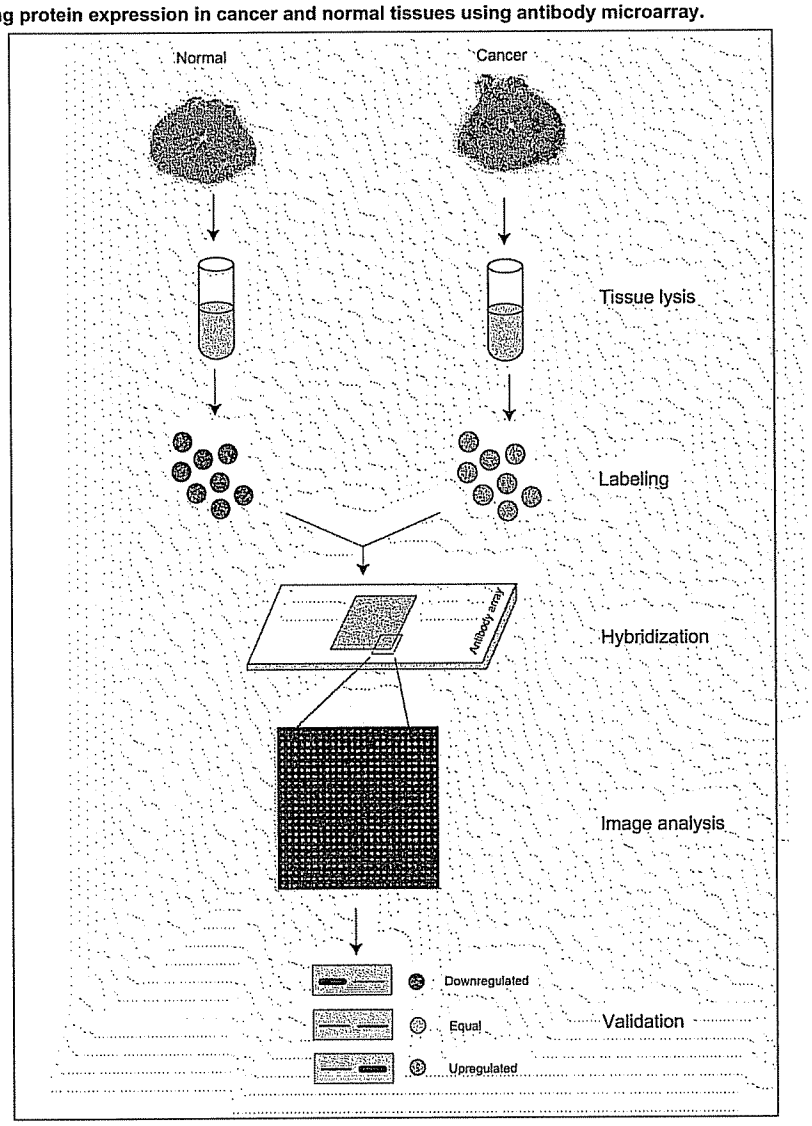
The principle underlying the development of antibody arrays is similar to the one used for antigen arrays. As shown in Figure 1, antibodies spotted on modified glass surfaces function as bait to capture cognate antigens from various biological samples. As in antigen arrays, the total proteins are extracted from the samples (cell line, tissue, sera or laser-captured cells). The proteins from the reference (untreated) and test (treated) samples are labeled with distinct fluorochromes; these are then mixed and hybridized on an antibody chip. The relative abundance of antigen in these samples is monitored by looking at the relative intensities of each reactive spot on the chip. The major issues in development of the antibody array involve proper orientation of the IgGs on the chip, maintenance of antibody conformation and reduction of cross-reactivity. The issues of orientation have been addressed using slides coated with various generic ligands such as protein G or biotin, or using affinity matrices such as Ni-NTA coated slides. The cross-reactivity of antibodies becomes less of an issue when using a two-color system.

Applications

The initial validation carried out by our research group and others showed antibody chips to be sensitive enough in the detection of extremely low amounts of antigen in complex solutions, similar to conventional techniques, such as ELISA and 2D electrophoresis. However, the advantage of antibody arrays lies in throughput, which allows for studying most of the proteins in a cell, against 2D gels, and the detection of only a fraction of a cell's proteome [2]. Using a two-color antibody array, a direct comparison of changes in protein expression could be made between normal and treated cells. To date, there are only a few reports using antibody arrays to profile protein expression in cells. Our group used a 1920 element antibody array consisting of 141 distinct antibodies to profile radiation-induced changes in protein levels in colon carcinoma cells [10••]. The data showed a number of novel associations between γ-radiation and apoptosis. Interestingly, the various regulators identified in the study could be assembled to define a pathway for radiation-induced apoptosis [10••]. Similarly, using an antibody chip comprising 368 antibodies and pure populations of cancer cells, Knezevic *et al* demonstrated cancer-specific alterations in levels of various proteins using biotin-based signal amplification [16••].

In addition to the above studies that used commercially available antibodies for profiling protein expression, one could also use a phage display library expressing antibody fragments to select candidates that are specifically expressed in cancers. Attempts in this direction were made by de Wildt *et al* who used an scFv library to select for antibodies against cell type-specific proteins [23••]. Thus, they were able to identify a subset of antibodies which recognized antigens specific to HeLa and yeast cell lysates [23••]. This technology could be applied to select for antibodies against various tumor markers, whose expression could be used to gauge tumor development, progression or invasion. Such a strategy also becomes useful in differentiating tumors that share common immunophenotypic characteristics.

Figure 1. Monitoring protein expression in cancer and normal tissues using antibody microarray.



Total proteins are extracted from cancer/normal tissues using detergents such as 1% NP40. The lysate is subjected to detergent removal using 1% Triton X-114 or extrigel beads. The protein content in each of the samples is estimated and equal amounts of protein are labeled using Cy5 or Cy3 NHS esters. The labeled proteins are separated from the free dye using gel filtration chromatography, mixed, concentrated and hybridized on an antibody chip. After hybridization, the slides are washed and scanned using an axon scanner. The scanned slides are analyzed and the relative levels of proteins in the cancer and normal samples are obtained. An upregulated protein appears as a red spot on the scanned image, while a downregulated protein appears green. Proteins showing equal expression in both the samples appear as yellow spots. The protein array data are validated using conventional immunoblot analysis.

In addition to looking at the protein profiles in cancer cells, antibody arrays are powerful tools enabling the study of levels of circulating proteins in sera. A clinically useful application in this area involves the profiling of chemokines in patient sera from various disease states. Chemokines are the chemical messengers secreted during the onset of inflammatory processes that transfer information between different cells in the body. Cancer could also be considered to be an inflammatory process and thus could involve the interplay between numerous cytokines. Routine methods of assaying chemokines rely on sandwich ELISA, which is sensitive but suffers from the drawback of being time consuming, especially in cases where numerous cytokines are to be monitored simultaneously. Thus a combination of sandwich ELISA and antibody arrays would give sensitivity as well as robustness to the assay system. A chemokine array designed on this rationale was used for profiling 35 cytokines, to understand the role of vitamin E in blocking tumor progression in liver epithelial cells [24•]. The authors found significant decreases in levels of epithelial cell-derived neutrophil-activity peptide-78, interleukin-1β, macrophage chemoattractant protein-1, RANTES, monokine

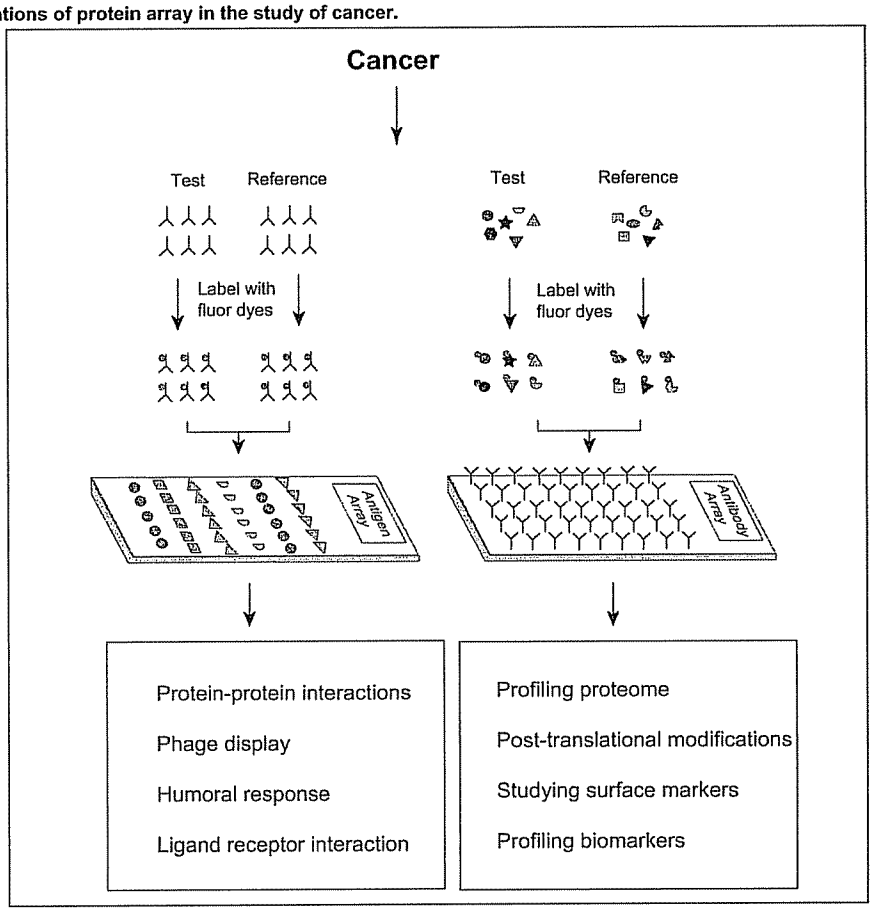
induced by interferon-γ and tumor necrosis factor-α in response to vitamin E treatment [24•]. One of the important issues in developing such high-throughput, antibody array-based chemokine detection would be to obtain biologically relevant sensitivity. This could be achieved by integrating rolling circle amplification as a mode of detection in the chemokine arrays [25••]. Such a system has been shown to have femtomolar sensitivity and a 3-fold log dynamic range in detection of various cytokines [25••]. Rolling circle amplification involves coupling an oligonucleotide primer to a universal antibody such as anti-biotin [26]. Once oligonucleotide-conjugated anti-biotin bound to its target on the chip, rolling circle amplification was initiated from the oligonucleotide primer using DNA polymerase, resulting in the generation of long concatenated sequences [26]. This concatemer was hybridized by fluorescently labeled oligonucleotides, resulting in signal amplification [25••].

In a parallel approach, the principles of flow cytometry and antibody arrays were combined to study expression of surface markers in leukemia [27•]. Routine diagnosis and classification of leukemia in the clinic rely on the use of a small subset of CD antigens. However, many leukemia and lymphomas share similar expression patterns for this limited group of antigens, and hence such an approach could lead to ambiguous results in many cases. Using antibody arrays, it is possible to immunotype this cancer based on detection of a larger set of CD antigens specific to the different types of leukemia. This would also allow multiplexing the expression for a large group of markers enabling a more reliable diagnosis of the disease. Belov *et al* used arrays containing antibodies to various CD antigens to immunophenotype various classes of leukemia [27•]. Using these antibody arrays, they were able to determine expression levels of approximately 50 CD antigens specific to leukocytes and leukemic cells [27•]. This approach thus allows for classification of closely related, phenotypically indistinct cancers.

Concluding remarks

Protein microarrays are powerful tools for studying global proteomic profiles of cells. As shown in Figure 2, antigen arrays and antibody arrays could be used in a variety of ways

Figure 2. Applications of protein array in the study of cancer.



Protein arrays have diverse applications in the study of cancer. Since the amount of analyte required for analysis is minute, different types of biological samples can be assayed. These include tissues, biological fluids such as serum, urine, seminal fluid and laser-captured cells. The antigen arrays could be used to study protein-protein interactions, phage display, monitor autoantibodies in patient sera against various cancer proteins and study ligand-receptor interactions. The antibody arrays could be used to profile protein expression in cancer cells, study post-translational modifications, classify cancers using multiplexed surface markers and profile serum biomarkers.

to study protein-protein interactions, cancer-specific humoral response, biomarker discovery and to profile inflammatory cytokines. This gives a better understanding of molecular events regulating development, survival and invasiveness of cancer. Such a wealth of proteomic information is difficult to obtain using any of the existing techniques due to their inability to adapt to high-throughput platforms, and in some cases, a narrow dynamic range. A combination of the proteomic data with the existing pool of genomic data would allow scientists to accurately predict cancer occurrence, design therapeutics to prevent the progression and invasion of cancer and thus reduce the incidence of death caused by this disease.

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